



# Regulation of $\text{Ca}^{2+}$ influx by a protein kinase C activator in chromaffin cells: differential role of P/Q- and L-type $\text{Ca}^{2+}$ channels

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## Abstract

Phorbol esters reduce depolarization-evoked  $\text{Ca}^{2+}$  influx in adrenal chromaffin cells, suggesting that voltage-sensitive  $\text{Ca}^{2+}$  channels (VSCCs) are inhibited by protein kinase C-mediated phosphorylation. We now address the possibility that L- and P/Q-type  $\text{Ca}^{2+}$  channel subtypes might be differentially involved in phorbol ester action. In bovine chromaffin cells, short-term (10 min) incubations with phorbol 12-myristate 13-acetate (PMA) inhibited early high  $\text{K}^{+}$ -evoked rises in cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and the early component of the depolarization-evoked  $\text{Mn}^{2+}$  quenching of fura-2 fluorescence in a dose-dependent manner ( $\text{IC}_{50}$ : 18 and 7 nM; maximal inhibitions: 45 and 48%, respectively). The protein kinase C inhibitor staurosporine (100 nM) reverted the inhibitory action of PMA. PMA (0.1–1  $\mu\text{M}$ ) inhibited the early and late phases of the ionomycin (2  $\mu\text{M}$ )-evoked  $[\text{Ca}^{2+}]_i$  transients by 14–23%.  $\omega$ -Agatoxin IVA, a blocker of P/Q-type  $\text{Ca}^{2+}$  channels, inhibited high  $\text{K}^{+}$ -evoked  $[\text{Ca}^{2+}]_i$  rises in a dose-dependent fashion ( $\text{IC}_{50}$  = 50 nM). In contrast, 0.1  $\mu\text{M}$   $\omega$ -conotoxin GVIA, a blocker of N-type channels, was without effect. A sizeable (< 45%) component of early  $\text{Ca}^{2+}$  influx persisted in the combined presence of  $\omega$ -agatoxin IVA (100 nM) and nitrendipine (1  $\mu\text{M}$ ). Simultaneous exposure to  $\omega$ -agatoxin IVA and PMA inhibited both the early  $[\text{Ca}^{2+}]_i$  transients and  $\text{Mn}^{2+}$  quenching to a much greater extent than each drug separately. Inhibition of the  $[\text{Ca}^{2+}]_i$  transients by nitrendipine and PMA did not significantly exceed that produced by PMA alone. It is concluded that phorbol ester-mediated activation of protein kinase C inhibits preferentially L-type VSCCs over P/Q type channels in adrenal chromaffin cells. However, the possibility cannot be ruled out that dihydropyridine-resistant, non-P/Q type channels might also be negatively regulated by protein kinase C. This may represent an important pathway for the specific control of VSCCs by protein kinase C-linked receptors, not only in paraneurons but presumably also in neurons and other excitable cells. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Adrenal medulla chromaffin cell;  $\text{Ca}^{2+}$  concentration, cytosolic free; Phorbol ester; Protein kinase C;  $\text{Ca}^{2+}$  channel, voltage-sensitive

## 1. Introduction

Adrenal chromaffin cells release catecholamines in response to acetylcholine, which is liberated from adjoining splanchnic nerve terminals. Acetylcholine acts on two membrane receptors, the nicotinic and muscarinic cholinergic receptors (Cheek et al., 1989; Burgoyne, 1991). The accepted sequence of events following agonist binding to the nicotinic receptor involves the activation of a non-selective receptor-operated channel, membrane depolariza-

tion, activation of voltage-sensitive  $\text{Ca}^{2+}$  channels, an increase in  $[\text{Ca}^{2+}]_i$  and the subsequent activation of the exocytotic machinery (Burgoyne, 1991). Agonist binding to the muscarinic receptor leads to the production of inositol 1,4,5-trisphosphate ( $\text{Ins}1,4,5\text{-P}_3$ ) and diacylglycerol, resulting in release of  $\text{Ca}^{2+}$  from internal stores and activation of protein kinase C (Berridge and Irvine, 1984). Protein kinase C is thought to increase the  $\text{Ca}^{2+}$  sensitivity of key proteins of the chromaffin cell by phosphorylating specific serine/threonine residues on these proteins (Terbush and Holz, 1990), resulting ultimately in the enlargement of the readily releasable pool of secretory granules (Gillis et al., 1996).

Protein kinase C-mediated phosphorylation processes are also known to influence voltage-sensitive  $\text{Ca}^{2+}$  chan-

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nels in several neuronal and non-neuronal cell types (Shearman et al., 1989). Protein kinase C activation either leads to the enhancement (Swartz, 1993; Yang and Tsien, 1993) or inhibition (Rane et al., 1989; Chik et al., 1996; Pemberton and Jones, 1997; Redman et al., 1997) of depolarization-evoked  $\text{Ca}^{2+}$  currents, depending on the cell type. In chromaffin cells, protein kinase C activation either has no effect (Burgoyne and Norman, 1984; Morita et al., 1985) or has opposite effects (Wakade et al., 1986; Pruss and Stauderman, 1988) on depolarization-evoked  $\text{Ca}^{2+}$  fluxes and catecholamine secretion depending on the species. For example, we have previously shown that 100–200 nM phorbol 12-myristate 13-acetate (PMA) inhibits depolarization-evoked catecholamine release and the underlying  $[\text{Ca}^{2+}]_i$  transients in bovine chromaffin cells (Sena et al., 1995). Protein kinase C activation has also been shown to inhibit voltage-sensitive  $\text{Ca}^{2+}$  fluxes in the closely related PC12 pheochromocytoma cell line (Di Virgilio et al., 1986; Bouron and Reber, 1994). Since voltage-sensitive  $\text{Ca}^{2+}$  channels are highly heterogeneous in different cell types, several studies have addressed the possibility that the various  $\text{Ca}^{2+}$  channel subtypes might be differentially regulated by the kinase (Cox and Dunlap, 1992; Yang and Tsien, 1993; Bouron and Reber, 1994). Bovine adrenal chromaffin cells are endowed with voltage-sensitive  $\text{Ca}^{2+}$  channels that resemble L-, N- and P/Q-types (Cena et al., 1983; Rosario et al., 1989; Bossu et al., 1991; Artalejo et al., 1992; Albillos et al., 1993; Duarte et al., 1993a,b; Lopez et al., 1994). While patch-clamp studies indicate that the dihydropyridine-sensitive L-type channels are controlled by the cAMP-dependent protein kinase (Artalejo et al., 1990), there is no information on the possible involvement of protein kinase C in the control of specific voltage-sensitive  $\text{Ca}^{2+}$  channel types in chromaffin cells.

In the present work, we used fura-2 fluorescence techniques to investigate the action of phorbol ester activation of protein kinase C on depolarization-evoked  $\text{Ca}^{2+}$  influx in bovine adrenal chromaffin cells. Specifically, by using selective toxin and non-toxin blockers of different voltage-sensitive  $\text{Ca}^{2+}$  channels subtypes, we have attempted at clarifying their possible differential role in phorbol ester action.

## 2. Materials and methods

### 2.1. Cell preparation and culture

Bovine adrenal glands were obtained from the local slaughterhouse. Adrenal medulla cells were isolated by collagenase digestion of the glands and purified on a Percoll density gradient essentially as described previously (Duarte et al., 1993a,b). Cells were cultured under a 5%  $\text{CO}_2$ /95% air humidified atmosphere in a 1:1 mixture of

Dulbecco's modified Eagle's medium/Ham's F-12 medium, buffered with 15 mM HEPES and 26 mM  $\text{NaHCO}_3$ , and supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ) and amphotericin B (250 ng/ml). Cells were kept in suspension culture in bacterial petri dishes (Nunc 240142) at a density of  $1.5 \times 10^6$  cells/30 ml for each dish.

### 2.2. $[\text{Ca}^{2+}]_i$ measurements

Chromaffin cells were loaded with the fluorescent  $\text{Ca}^{2+}$  indicator fura-2 (Grynkiewicz et al., 1985) essentially as described previously (Duarte et al., 1993a,b). The final pellet of loaded cells was stored on ice to minimize fura-2 leakage. Prior to each experiment, an aliquot of chromaffin cells ( $0.75 \times 10^6$  cells/ml, final density) was transferred to a cuvette and preincubated in physiological salt solution for 5 min at 30°C. This solution had the following composition (mM): 130 NaCl, 5 KCl, 2  $\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 10 glucose and 15 HEPES-Na (pH 7.35). The fluorescence was monitored using a computer-assisted spectrofluorometer (Fluoromax, Spex Industries, Edison, NJ, USA), with excitation at 340 nm and emission at 510 nm, using 5- and 10-nm slits, respectively. Detailed procedures for the recording and calibration of fluorescence data were essential as reported previously (Duarte et al., 1993a,b). The fluorescence intensities were automatically converted into  $[\text{Ca}^{2+}]_i$  values using the calibration equation for single excitation measurements and taking the dissociation constant of the fura-2/ $\text{Ca}^{2+}$  complex as 224 nM (Grynkiewicz et al., 1985).

### 2.3. Fluorescence quenching experiments

$\text{Mn}^{2+}$  influx was monitored from chromaffin cell suspensions by subjecting the cells to 50  $\mu\text{M}$   $\text{MnCl}_2$  in the absence of external  $\text{Ca}^{2+}$  while recording the fura-2 fluorescence at the dye isosbestic point (360 nm), as described (Sena et al., 1995). Under these conditions, any decrease in fluorescent intensity (quenching) reflects the extent to which fura-2 is complexed by intracellular  $\text{Mn}^{2+}$  (and thus the extent of  $\text{Mn}^{2+}$  influx), irrespectively of the magnitude of the  $[\text{Ca}^{2+}]_i$  changes that may occur simultaneously (Hallam et al., 1988; Jacob, 1990).

### 2.4. Statistical analysis

Values are expressed as mean  $\pm$  S.D. throughout the text and figures. Statistical significance of differences between mean values was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test, as appropriate. Differences between non-normalized responses obtained at each drug concentration and control responses were considered to be statistically significant at the 95% confidence level.

## 2.5. Materials

Collagenase B was obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany). Antibiotics and fetal calf serum were from Biological Industries (Beth Haemek, Israel). Percoll was from Pharmacia LKB (Uppsala, Sweden). Fura-2/AM ({1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid pentaacetoxymethyl ester}) was from Molecular Probes (Eugene, OR, USA). Nitrendipine was purchased from Sandoz Laboratories (Basle, Switzerland).  $\omega$ -Agatoxin IVA was from Peptide Institute (Osaka, Japan).  $\omega$ -Conotoxin GVIA was from Peninsula Laboratories (Belmont, CA, USA). PMA and staurosporine were from Calbiochem (San Diego, CA, USA). All other reagents were either from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Concentrated stock solutions of nitrendipine, PMA and staurosporine were prepared in DMSO, sampled in small aliquots, and kept at  $-20^{\circ}\text{C}$  prior to use. The concentrated stock solution of  $\omega$ -agatoxin IVA and  $\omega$ -conotoxin GVIA was prepared in distilled water sampled in small aliquots and stored as above.

## 3. Results

We have recorded the  $[\text{Ca}^{2+}]_i$  from suspensions of bovine chromaffin cells using the fluorescent  $\text{Ca}^{2+}$  indicator fura-2. Average resting  $[\text{Ca}^{2+}]_i$  in these experiments was  $138 \pm 28$  nM ( $\pm$ S.D.,  $n = 21$  experiments). Raising medium  $\text{K}^+$  concentration from 5 to 50 mM evoked a sharp  $[\text{Ca}^{2+}]_i$  increase, followed by a relatively slow relaxation towards a plateau (leftmost trace in Fig. 1A). This  $[\text{Ca}^{2+}]_i$  rise reflects primarily  $\text{Ca}^{2+}$  influx through voltage-sensitive  $\text{Ca}^{2+}$  channels (Rosario et al., 1989).

Protein kinase C can be activated by short-term incubations with phorbol esters such as PMA (Castagna et al., 1982). In fact, we have recently demonstrated the presence of three protein kinase C isoforms (protein kinase C- $\alpha$ , - $\varepsilon$  and - $\zeta$ ) in bovine chromaffin cells and that both protein kinase C- $\alpha$  and - $\varepsilon$  undergo rapid translocation from cytosol to membrane following short-term treatments with PMA (Sena et al., 1996). In the following experiments, we assessed the effect of PMA on the depolarization-evoked  $[\text{Ca}^{2+}]_i$  transients by comparing the size of these transients in the presence and absence of the phorbol ester.

Fig. 1A and B shows that short (10 min) incubations with PMA reduced the peak of the  $\text{K}^+$ -evoked  $[\text{Ca}^{2+}]_i$  rise in a dose-dependent fashion. The PMA inhibition plateaued off at 1  $\mu\text{M}$  PMA and did not exceed 45% of the control response (Fig. 1B, diamonds). In addition, Fig. 1B shows that the drug concentration required to cause 50% of the maximal inhibition ( $\text{IC}_{50}$ ) was 17.8 nM. We have also assessed the inhibitory effect of PMA upon the  $[\text{Ca}^{2+}]_i$

rise recorded at a later stage (2 min after stimulation with high  $\text{K}^+$ ). The extent of the inhibition calculated in this way (Fig. 1B, circles) was more pronounced at all PMA concentrations tested, with maximal inhibition amounting to 76% of the control response. The  $\text{IC}_{50}$  value for the inhibition of the late response (18.2 nM) was, however, not significantly different from the value calculated for the peak response.

Previous studies, carried out on single chromaffin cells, showed that the inhibitory effect of 200 nM PMA on high  $\text{K}^+$ -evoked  $[\text{Ca}^{2+}]_i$  transients was near-maximal after 10-min incubations with the phorbol ester (Sena et al., 1995). We have observed in the present study that the inhibitory effect of PMA (0.5  $\mu\text{M}$ ) became maximal for incubation periods intermediate between 5 and 10 min (data not shown).

The following experiments were designed to assess the possibility that PMA might indeed inhibit voltage-sensitive  $\text{Ca}^{2+}$  channels in chromaffin cells. To this end, a small amount (50  $\mu\text{M}$ ) of the  $\text{Ca}^{2+}$  channel surrogate  $\text{Mn}^{2+}$  (Stauderman and Pruss, 1989; Jacob, 1990) was added to the cells in the absence of external  $\text{Ca}^{2+}$  and the fura-2 fluorescence was recorded at the dye isosbestic point (360 nm). The fluorescence baseline was stable in the absence of  $\text{Mn}^{2+}$  (not shown) and started to decline upon addition of the divalent cation (leftmost superimposed traces in Fig. 1C). Since  $\text{Mn}^{2+}$  is a strong quencher of fura-2 fluorescence (Hallam et al., 1988), the latter process likely originates from basal  $\text{Mn}^{2+}$  influx through  $\text{Ca}^{2+}$ -permeant channels. Raising medium  $\text{K}^+$  concentration to 50 mM caused a rapid and pronounced fluorescence decrease, indicating enhanced access of  $\text{Mn}^{2+}$  to the cytoplasm (Fig. 1C, trace labelled 'control'). Fig. 1C also shows that the  $\text{K}^+$ -evoked quenching of fura-2 fluorescence was impaired in a dose-dependent manner by 10-min incubations with PMA.

Extracellular  $\text{Mn}^{2+}$  concentration is likely to remain constant throughout the experiment and, therefore,  $\text{Mn}^{2+}$  influx through membrane channels can, to a first approximation, be treated as a zero order process. Intracellular accumulation of the divalent cation and the concomitant quenching of fura-2 fluorescence would then be expected to proceed linearly along the first few seconds after stimulation. We have therefore fitted the early components of the quenching traces to a linear function and plotted the respective slopes as a function of PMA concentration (Fig. 1D). PMA decreased the slopes in a dose-dependent fashion ( $\text{IC}_{50} = 7$  nM), the effect being maximal at around 0.1  $\mu\text{M}$ . Interestingly, the latter concentration is at least one order of magnitude less than the maximal PMA concentration required to minimize the extent of the overall quenching process, as inferred from the difference between the fluorescence recorded in the absence of any stimulus (trace labelled 'no stimulus' in Fig. 1C) and the fluorescence recorded at each PMA concentration, approximately 1 min after  $\text{K}^+$  stimulation. It is also apparent from Fig. 1D that,

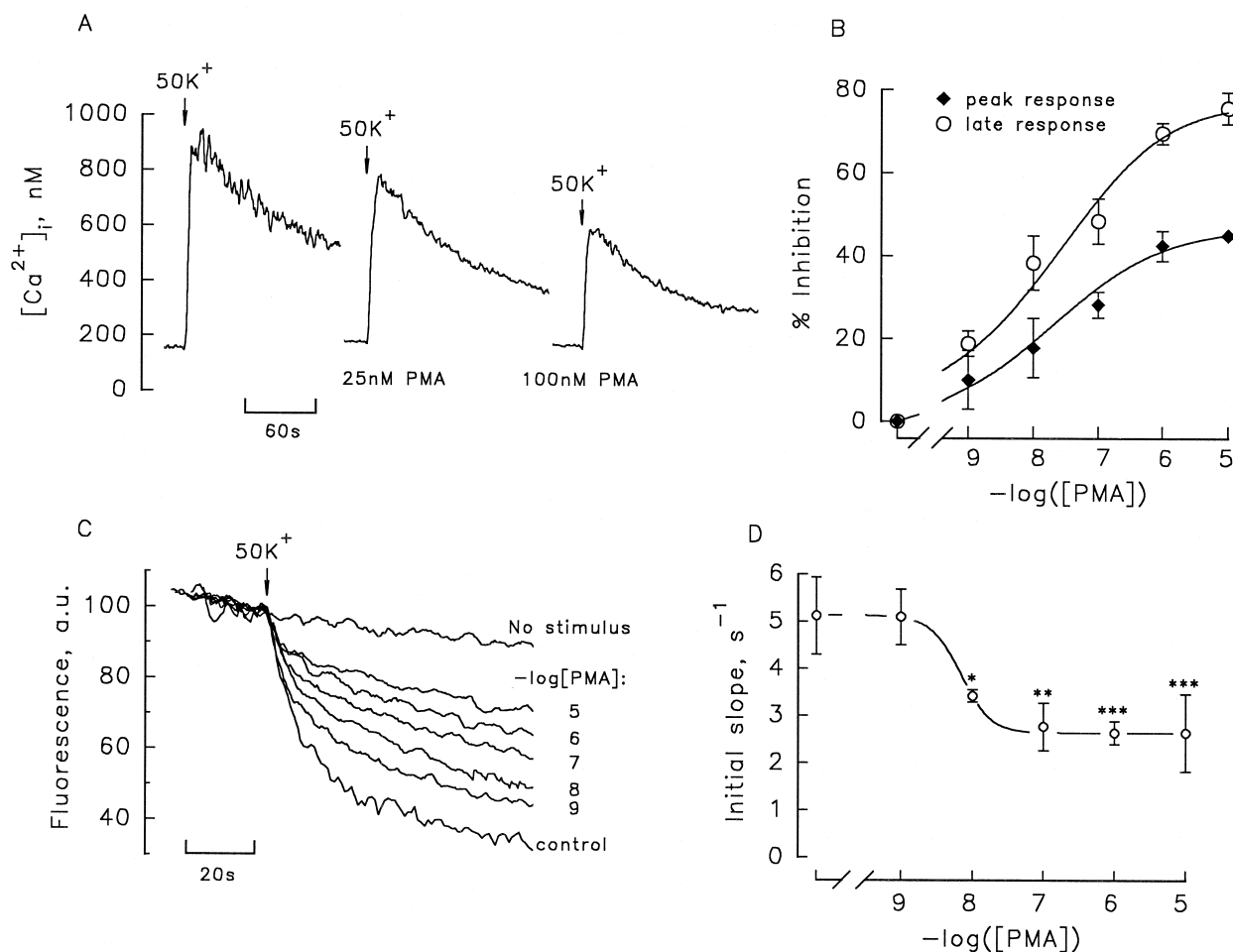


Fig. 1. Dose-dependent inhibition of depolarization-evoked  $[Ca^{2+}]_i$  transients and  $Mn^{2+}$  influx by the protein kinase C activator PMA. (A) The cells were stimulated with 50 mM KCl ( $50K^+$ ) as denoted by the arrows, in the absence (leftmost trace) and presence of various concentrations of PMA (remaining traces). Cells were exposed to PMA for 10 min prior to stimulation in all cases. (B) PMA-mediated inhibition of the  $[Ca^{2+}]_i$  transients as a function of PMA concentration. The percentage of inhibition was calculated as  $I = 100 \times (A_C - A_P)/A_C$ , where  $A_C$  and  $A_P$  stand for the  $[Ca^{2+}]_i$  changes measured from the control experiment and from the experiments carried out in the presence of PMA, respectively. Diamonds and circles represent calculations based on the peak  $[Ca^{2+}]_i$  responses and on the  $[Ca^{2+}]_i$  rises recorded at a later stage (2 min after stimulation with high  $K^+$ ), respectively. The absolute average peak  $[Ca^{2+}]_i$  changes calculated from the control experiments were  $626 \pm 6$  nM ( $n = 10$  measurements). (C) The cells were exposed to 50  $\mu$ M  $Mn^{2+}$  in the absence of external  $Ca^{2+}$  and the fura-2 fluorescence was recorded at the dye isosbestic point (360 nm), as described in Section 2. Superimposed fluorescence traces depicting the quenching of fura-2 fluorescence evoked by stimulating the cells with 50 mM KCl in the absence of PMA (trace labelled 'control') and the effects on this quenching of 10-min incubations with various PMA concentrations. The 'no stimulus' trace depicts an experiment where the cells have not been challenged with high  $K^+$ . The original fluorescence traces were normalized to the fluorescence recorded immediately prior to  $K^+$  stimulation (taken as 100 arbitrary units). (D) The fluorescence decays recorded 2–4 s after  $K^+$  stimulation were fitted to a linear function and the respective slopes plotted vs. the logarithm of PMA concentration. All linear fits had correlation coefficients in the range 0.95–0.99. The leftmost point in the plot denotes the average slope calculated from control traces (no PMA). Data are presented as mean  $\pm$  S.D. ( $n = 8$ –10 measurements). Statistical significance of differences between control responses and responses obtained at each drug concentration: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

similar to the effect of the phorbol ester on the peak of the  $K^+$ -evoked  $[Ca^{2+}]_i$  transients, high PMA concentrations failed to suppress the  $K^+$ -evoked early quenching of fura-2 fluorescence (the initial slopes measured at 1–10  $\mu$ M PMA were approximately 3 s<sup>-1</sup>, i.e., significantly above 0.1 s<sup>-1</sup>, the typical slope measured from the 'no stimulus' traces). Indeed, the maximal PMA inhibition assessed from Fig. 1D did not exceed 48% of control.

We have assessed the effect of 100 nM PMA on the depolarization-evoked  $[Ca^{2+}]_i$  transients in the absence and presence of staurosporine, a non-selective inhibitor of

serine/threonine protein kinases including protein kinase C (Rüegg and Burgess, 1989). Staurosporine reduced the peak of the  $K^+$ -evoked  $[Ca^{2+}]_i$  rise in a dose-dependent fashion ( $IC_{50} = 56$  nM; Fig. 2C, see panel A for an example) but failed to affect the  $[Ca^{2+}]_i$  rise recorded 2 min after stimulation (Fig. 2A and B). Importantly, staurosporine treatment impaired the inhibitory action of PMA both on the early and late  $[Ca^{2+}]_i$  responses. In fact, the size of the  $[Ca^{2+}]_i$  transients recorded in 100 nM staurosporine plus PMA approached that measured in control (Fig. 2A and B).

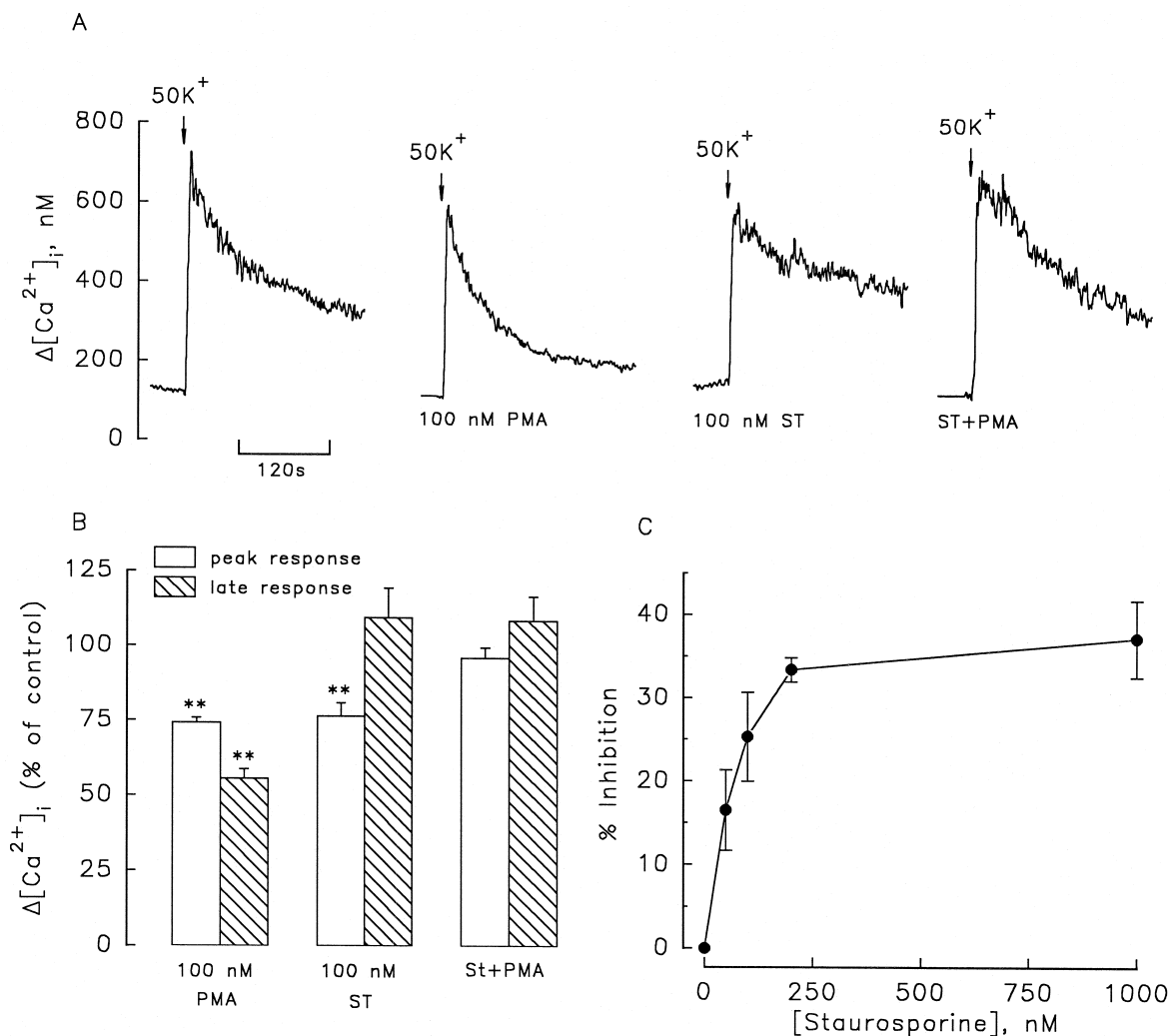


Fig. 2. Reversal of the inhibitory action of PMA by the protein kinase C inhibitor staurosporine. (A) The cells were stimulated with 50 mM KCl (50 K<sup>+</sup>) as denoted by the arrows, in the absence (leftmost trace) and presence of PMA, staurosporine (ST) and 100 nM PMA + 100 nM staurosporine (remaining traces). Cells were exposed to PMA and staurosporine for 10 min prior to stimulation in all cases. (B) PMA- and staurosporine-mediated inhibition of the  $[Ca^{2+}]_i$  transients. Columns represent average  $[Ca^{2+}]_i$  changes after 10 min in the presence of 100 nM PMA (PMA), 100 nM staurosporine (ST) and 100 nM PMA + 100 nM staurosporine (ST + PMA), normalized to the corresponding control change ( $[Ca^{2+}]_i$  changes evoked by 50 mM KCl alone; absolute peak control response:  $495 \pm 39$  nM,  $n = 12$  measurements). Empty and dashed columns correspond to measurements done at the peak  $[Ca^{2+}]_i$  responses and 2 min after high K<sup>+</sup> stimulation, respectively. Data from the experiment depicted in (A) and from five similar experiments, each performed in duplicate ( $n = 12$  measurements). (C) Dose-dependent inhibition of the high K<sup>+</sup>-evoked  $[Ca^{2+}]_i$  transients by staurosporine. The percentage of inhibition was calculated as explained in the legend to Fig. 1B. Data from the experiment depicted in (A) and from three similar experiments, each performed in duplicate ( $n = 8$  measurements). Data are presented as mean  $\pm$  S.D. Statistical significance of differences between non-normalized test responses and control responses: \*\*  $P < 0.01$ . In (B) differences between late  $[Ca^{2+}]_i$  responses obtained in staurosporine alone and staurosporine + PMA failed to reach statistical significance ( $P > 0.05$ ). The corresponding differences between peak  $[Ca^{2+}]_i$  responses were found to be statistically significant.

Since the K<sup>+</sup>-evoked  $[Ca^{2+}]_i$  transients reflect a complex interplay between depolarization-induced Ca<sup>2+</sup> influx and intracellular Ca<sup>2+</sup> buffering, the acute PMA inhibition attributed above to inhibition of voltage-sensitive Ca<sup>2+</sup> channels can conceivably be compounded by stimulation of Ca<sup>2+</sup> sequestering and/or extrusion mechanisms. To examine the buffering hypothesis, we have investigated the effect of PMA on the  $[Ca^{2+}]_i$  transients brought about by the Ca<sup>2+</sup> ionophore ionomycin. Ionomycin (2  $\mu$ M) evoked a sharp  $[Ca^{2+}]_i$  rise, followed by a relaxation towards a plateau (Fig. 3A). While the initial rise is seemingly due to

Ca<sup>2+</sup> release from internal stores, the plateau response appears to be primarily supported by ionophore-mediated Ca<sup>2+</sup> influx through the plasma membrane (Duarte and Rosario, 1989; Purkiss and Willars, 1996). Fig. 3A and B shows that, on average, acute exposure to 0.1  $\mu$ M PMA inhibited both the peak and the plateau responses by 14 and 22%, respectively (the average inhibitions relative to 1  $\mu$ M PMA were 21 and 23%, respectively). PMA (0.1–1  $\mu$ M) did not affect the kinetics of the  $[Ca^{2+}]_i$  decay from peak to plateau, as assessed by the calculation of the respective time constants (Fig. 3C).

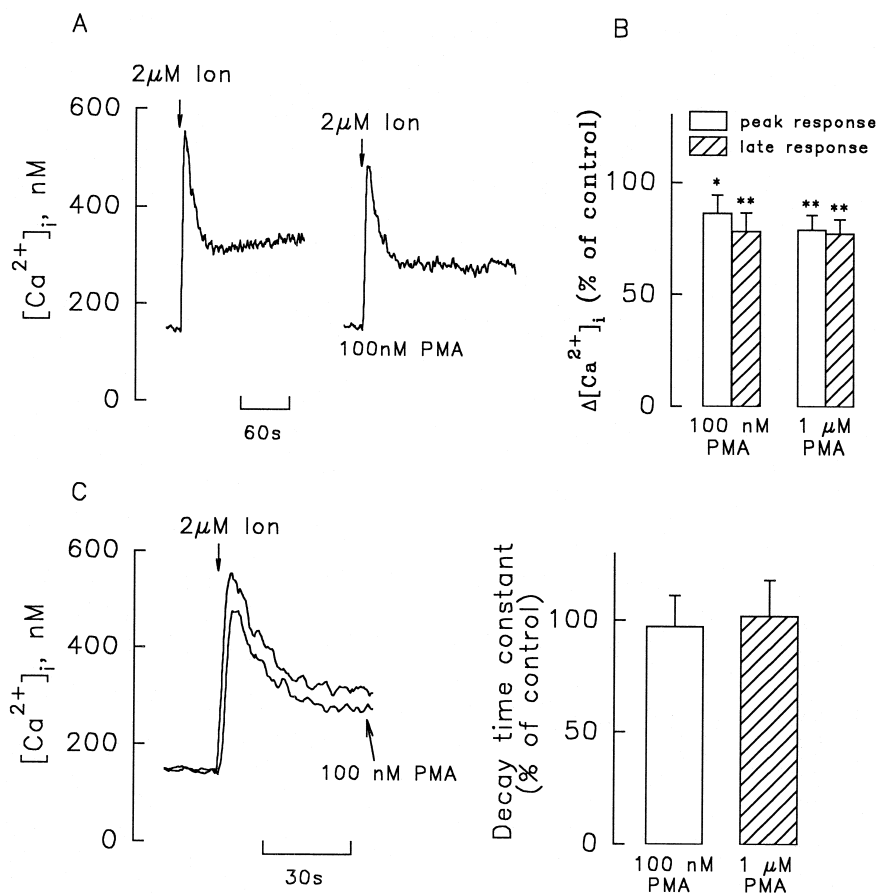


Fig. 3. Modulation of ionomycin-evoked  $[Ca^{2+}]_i$  transients by short-term incubations with PMA. (A) The cells were stimulated with ionomycin (Ion) as denoted by the arrows, in the absence (leftmost trace) and presence of PMA. Cells were exposed to the protein kinase C activator for 10 min prior to stimulation. (B) Columns represent average  $[Ca^{2+}]_i$  changes after 10 min in the presence of PMA (0.1 and 1  $\mu M$ ), normalized to the corresponding control change ( $[Ca^{2+}]_i$  changes evoked by 2  $\mu M$  ionomycin alone; absolute peak control response:  $335 \pm 21$  nM,  $n = 14$  measurements). Empty and dashed columns correspond to measurements done at the peak of the ionomycin responses and 2 min after ionophore addition, respectively. Data from the experiment depicted in (A) and from six similar experiments, each performed in duplicate ( $n = 14$  measurements). (C) Effect of ionomycin on an expanded time basis (upper trace: control; lower trace: 10-min incubation with PMA). The decay portion of the  $[Ca^{2+}]_i$  transients was fitted to a single exponential decay function ( $F = F_1 + F_2 \exp(-t/\tau)$ , where  $F_1$  and  $F_2$  are constants,  $t$  is time and  $\tau$  is the decay time constant). The columns represent the time constants obtained from the best fits to the data in the presence of PMA, normalized to the corresponding control values (decay time constant relative to stimulation with ionomycin alone; absolute control time constant:  $11.4 \pm 3.0$  s,  $n = 14$  measurements). Data from the experiment depicted to the left and from six similar experiments, each performed in duplicate ( $n = 14$  measurements). Data are presented as mean  $\pm$  S.D. Statistical significance of differences between non-normalized responses recorded in PMA and control responses: \*  $P < 0.05$ ; \*\*  $P < 0.01$ .

The PMA experiments depicted in Fig. 3 suggest that the phorbol ester stimulates  $Ca^{2+}$  sequestering and/or extrusion mechanisms in chromaffin cells, and that this might account partially for the PMA-mediated inhibition of the high  $K^+$ -evoked  $[Ca^{2+}]_i$  transients. However, because the inhibition of the ionomycin-evoked  $[Ca^{2+}]_i$  transients did not exceed 23%, i.e., significantly less than the PMA (1  $\mu M$ ) inhibition of the depolarization-evoked transients (43% and 70% for the peak and late phases, respectively), the latter is likely to be dominated by inhibition of voltage-sensitive  $Ca^{2+}$  channels.

Elucidating the role of different  $Ca^{2+}$  channel subtypes in the inhibitory effect of PMA on stimulated  $Ca^{2+}$  influx was a primary aim of this work. This involved the use of toxin and other specific blockers of  $Ca^{2+}$  channels subtypes present in chromaffin cells.  $\omega$ -Agatoxin IVA is

admittedly specific for P-type channels at low concentrations ( $< 10$  nM); at higher concentrations the toxin has been reported to block both P- and Q-type channels (Wheeler et al., 1994). Fig. 4A and B shows that  $\omega$ -agatoxin IVA reduced the high  $K^+$ -evoked peak  $[Ca^{2+}]_i$  rise in a dose-dependent way ( $IC_{50} = 50$  nM). Maximal inhibition of approximately 32% was attained at 100 nM (the concentration used to block P/Q type channels in the remainder of this study). In contrast, using a fresh toxin preparation and identical experimental conditions, parallel experiments demonstrated that  $\omega$ -conotoxin GVIA, a blocker of N-type channels (Fox et al., 1987; McCleskey et al., 1987), did not affect the high  $K^+$ -evoked  $[Ca^{2+}]_i$  rises (Fig. 4C). Indeed, the peak  $[Ca^{2+}]_i$  rise remaining in the presence of the toxin amounted to  $96 \pm 11\%$  ( $n = 3$  experiments) of that obtained in control. This confirms

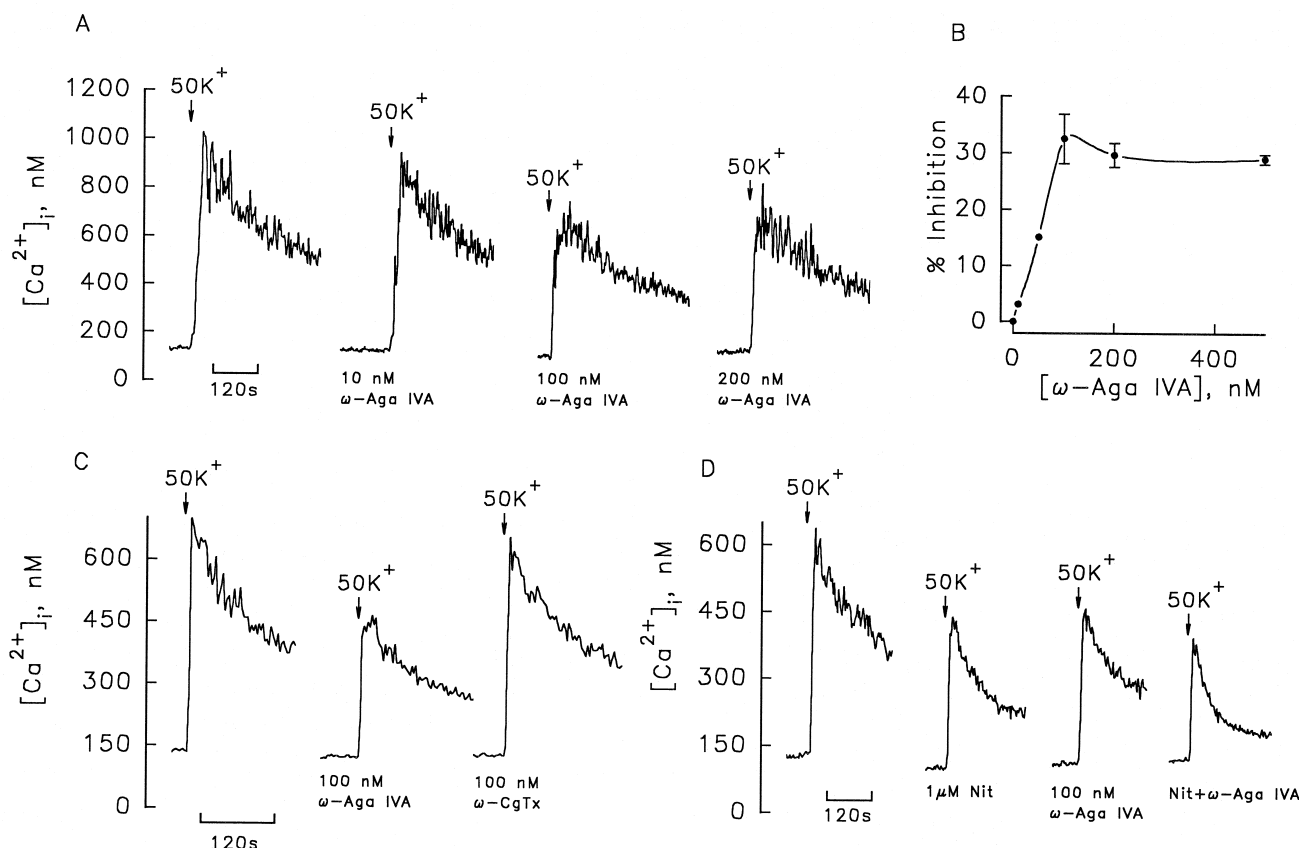


Fig. 4. Effects of different  $\text{Ca}^{2+}$  channel blockers on depolarization-evoked  $[\text{Ca}^{2+}]_i$  transients. (A) Cells were stimulated with 50 mM KCl ( $50 \text{ K}^+$ ) as denoted by the arrows, in the absence (leftmost trace) and presence of various concentrations of  $\omega$ -agatoxin IVA ( $\omega$ -Aga IVA, remaining traces). (B) Dose-dependent inhibition of the high  $\text{K}^+$ -evoked  $[\text{Ca}^{2+}]_i$  transients by  $\omega$ -agatoxin IVA. The percentage of inhibition was calculated as explained in the legend to Fig. 1B. Data from the experiment depicted in (A) and from four similar experiments, each performed in duplicate ( $n = 10$  measurements). (C) Lack of effect of  $\omega$ -conotoxin GVIA ( $\omega$ -Cg Tx) and contrast with the inhibitory effect  $\omega$ -agatoxin IVA in the same series of experiments. (D) Effects of combined exposures to  $\omega$ -agatoxin IVA (100 nM) and nitrendipine (Nit, 1  $\mu\text{M}$ ) and comparison with those of  $\omega$ -agatoxin IVA or nitrendipine alone. Cells were exposed to the toxins and nitrendipine for 10 min prior to stimulation in all cases. Experiments depicted in (C) and (D) are representative of two and four similar experiments, respectively. Data in (B) are presented as mean  $\pm$  S.D. (the size of the error bars is smaller than the symbols for 10 and 50 nM  $\omega$ -agatoxin IVA).

previous results from our laboratory (Rosario et al., 1989; Duarte et al., 1993a) (but see Section 4 for the assessment of results from other groups). The lack of effect of  $\omega$ -conotoxin GVIA, reported in the present study, is unlikely to be accounted for by either the specific concentration (0.1  $\mu\text{M}$ ) or incubation time (10 min) used in the experiments. Indeed, the toxin concentration used in our study is at least two orders of magnitude higher than the reported  $K_d$  for the low-affinity binding site in chromaffin cells (Ballesta et al., 1989). In addition, we have previously reported that 60-min incubations with 0.5  $\mu\text{M}$   $\omega$ -conotoxin GVIA did not affect high  $\text{K}^+$ -induced  $^{45}\text{Ca}^{2+}$  uptake in chromaffin cells (Duarte et al., 1993a).

The dihydropyridine nitrendipine, a blocker of L-type channels, reduced the high  $\text{K}^+$ -evoked peak  $[\text{Ca}^{2+}]_i$  rise by  $17 \pm 1\%$  ( $n = 5$  experiments; a representative experiment is shown in Fig. 4D). At the concentration used (1  $\mu\text{M}$ ), nitrendipine is expected to cause near-maximal blockade of  $\text{Ca}^{2+}$  influx through these channels. Indeed,

we have observed in the course of the present experiments that 0.1, 1 and 10  $\mu\text{M}$  nitrendipine inhibited high  $\text{K}^+$  (30 mM)-induced  $^{45}\text{Ca}^{2+}$  uptake by 48%, 54% and 58%, respectively (C.M. Sena and L.M. Rosário, unpublished observations). Furthermore, 1  $\mu\text{M}$  is about 1000 times greater than the  $K_d$  for nitrendipine binding to bovine adrenomedullary membrane fragments (Garcia et al., 1984; Ballesta et al., 1989). Somewhat unexpectedly, combined exposure to  $\omega$ -agatoxin IVA and nitrendipine did not suppress the high  $\text{K}^+$ -evoked  $[\text{Ca}^{2+}]_i$  rises and, instead, a sizeable component of early  $\text{Ca}^{2+}$  influx remained in the presence of both blockers (Fig. 4D). Indeed, the peak  $[\text{Ca}^{2+}]_i$  rise remaining in the presence of  $\omega$ -agatoxin IVA plus nitrendipine amounted to  $43 \pm 4\%$  of that obtained in control (vs.  $68 \pm 3\%$  and  $83 \pm 6\%$  for  $\omega$ -agatoxin IVA and nitrendipine alone in this series of experiments, respectively;  $n = 5$ ).

We and others have previously observed that PMA inhibits the  $[\text{Ca}^{2+}]_i$  rise evoked by the L-type  $\text{Ca}^{2+}$  chan-

nel agonist Bay K 8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridinecarboxylic acid methyl ester) (Kley, 1988; Sena et al., 1995), suggesting that this channel type might be a major target for the inhibitory action of the phorbol ester. To either support or rule out this hypothesis, experiments have now been carried out where the effect of combined applications of PMA and nitrendipine on the depolarization-evoked  $[Ca^{2+}]_i$  transients was compared with the effects of separate applications of each drug.

Fig. 5A and B shows again that 1  $\mu$ M nitrendipine depressed the high  $K^+$ -evoked  $[Ca^{2+}]_i$  rise, the inhibitory effect being however significantly more pronounced 2 min after stimulation than at the peak (39% and 17% of

control, respectively). Furthermore, PMA depressed the peak and late phases of the  $[Ca^{2+}]_i$  transients to a larger extent than nitrendipine. It is also noteworthy that, in most experiments, the magnitude of the  $[Ca^{2+}]_i$  transients recorded in the presence of PMA alone was similar to that in the presence of nitrendipine plus PMA (Fig. 5A); in some experiments, the combination of drugs produced a somewhat larger inhibition than PMA alone; however, the average inhibitory effect of PMA was not significantly different from that of nitrendipine plus PMA, regardless of whether this referred to the peak or to the late phase of the  $[Ca^{2+}]_i$  transients (Fig. 5B). This result is consistent with PMA exerting a pronounced inhibitory effect upon L-type  $Ca^{2+}$  channels.

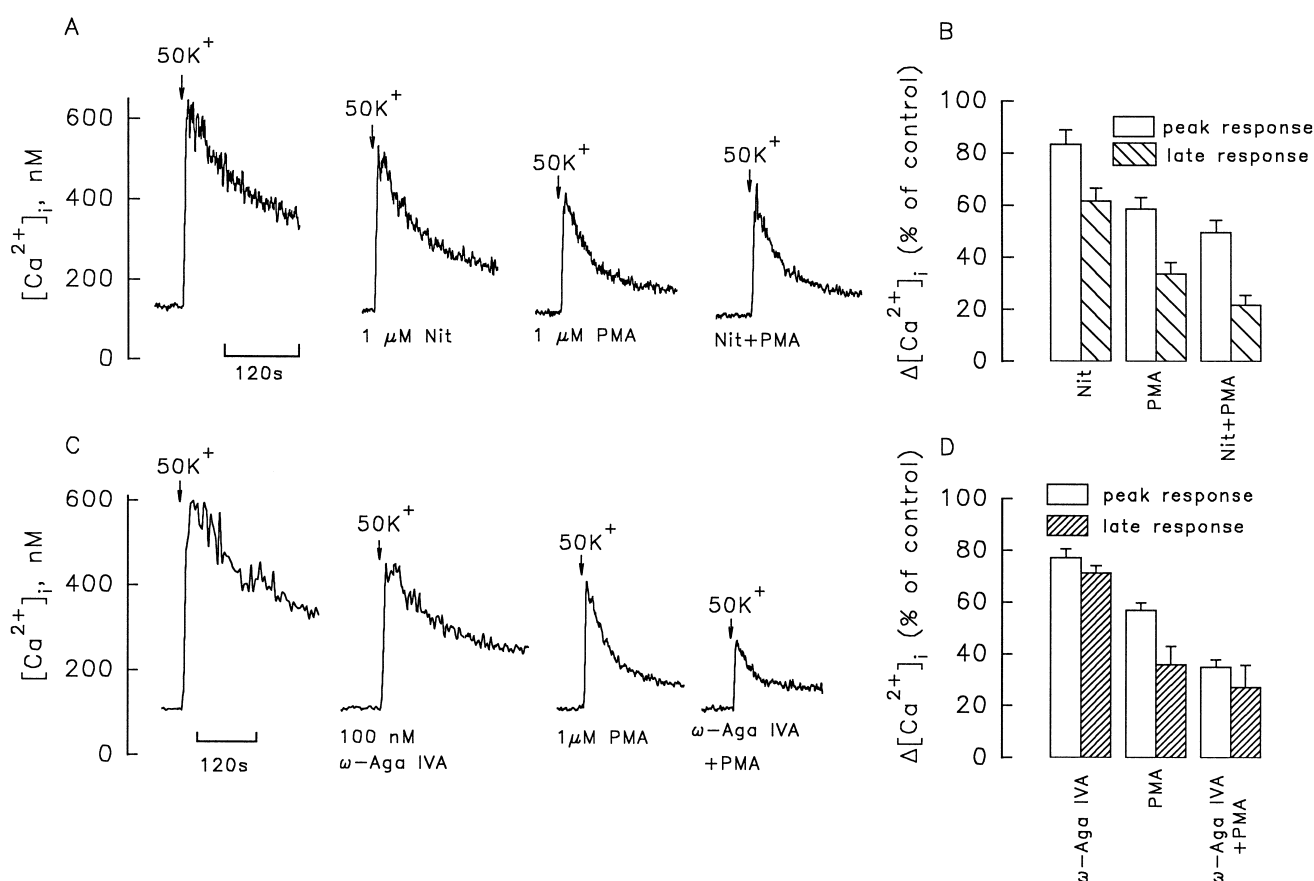


Fig. 5. Differential inhibitory effect of PMA and  $Ca^{2+}$  channel blockers upon depolarization-evoked  $[Ca^{2+}]_i$  transients. (A) Cells were stimulated with 50 mM KCl ( $50 K^+$ ) as denoted by the arrows, in the absence (leftmost trace) and presence of nitrendipine (Nit), PMA or 1  $\mu$ M nitrendipine + 1  $\mu$ M PMA (remaining traces). Cells were exposed to PMA and nitrendipine for 10 min prior to stimulation. (B) Columns labelled 'Nit', 'PMA' and 'Nit + PMA' represent average  $[Ca^{2+}]_i$  changes after 10 min in the presence of each drug (or combination of drugs), normalized to the corresponding control change ( $[Ca^{2+}]_i$  changes evoked by 50 mM KCl alone; absolute peak control response:  $482 \pm 32$  nM,  $n = 12$  measurements). Empty and dashed columns correspond to measurements done at the peak of the high  $K^+$  responses and 2 min after  $K^+$  stimulation, respectively. Data from the experiment depicted in (A) and from five similar experiments, each performed in duplicate ( $n = 12$  measurements). (C) Essentially as for (A), except that nitrendipine was replaced for  $\omega$ -agatoxin IVA ( $\omega$ -Aga IVA). (D) As for (B), except that nitrendipine was replaced for  $\omega$ -agatoxin IVA. Absolute peak control response:  $689 \pm 49$  nM ( $n = 10$  measurements). Data from the experiment depicted in (C) and from four similar experiments, each performed in duplicate ( $n = 10$  measurements). Data are presented as mean  $\pm$  S.D. Differences between non-normalized responses obtained in the presence of each drug (or combination of drugs) and control responses were found to be statistically significant. Differences between data obtained in PMA alone and nitrendipine + PMA failed to reach statistical significance ( $P > 0.05$ ). Differences between peak response data obtained in PMA alone and  $\omega$ -agatoxin IVA + PMA were found to be statistically significant. In contrast, the corresponding late response data failed to reach statistical significance ( $P > 0.05$ ).



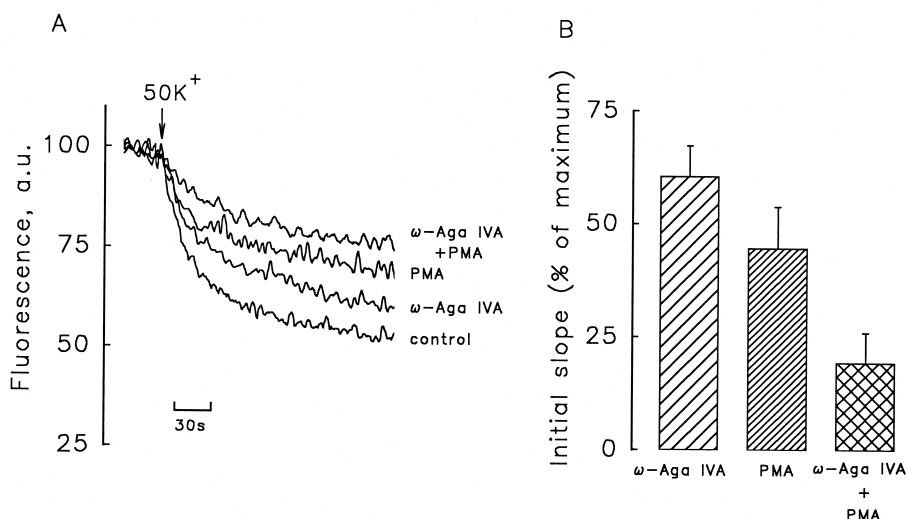


Fig. 6. Differential inhibitory effect of PMA and  $\omega$ -agatoxin IVA upon depolarization-evoked  $\text{Mn}^{2+}$  influx. (A) Essentially as for Fig. 1C, except that the drugs were 100 nM  $\omega$ -agatoxin IVA (trace labelled  $\omega$ -Aga IVA), 1  $\mu\text{M}$  PMA (trace labelled PMA) and 100 nM  $\omega$ -agatoxin IVA + 1  $\mu\text{M}$  PMA (trace labelled  $\omega$ -Aga IVA + PMA). (B) Average early decay slopes (see legend to Fig. 1D), determined from experiments where the cells were exposed to 100 nM  $\omega$ -agatoxin IVA, 1  $\mu\text{M}$  PMA and 100 nM  $\omega$ -agatoxin IVA + 1  $\mu\text{M}$  PMA. Data from the experiment depicted in (A) and five similar experiments. Data have been normalized to the maximum response (early decay slope determined from control experiments; absolute control slope:  $3.9 \pm 1.8 \text{ s}^{-1}$ ,  $n = 12$  measurements). The data are presented as mean  $\pm$  S.D. ( $n = 12$  measurements). Differences between non-normalized responses obtained in the presence of each drug (or combination of drugs) and control responses were found to be statistically significant. Differences between the data obtained in PMA alone and  $\omega$ -agatoxin IVA + PMA were also statistically significant.

The possibility that PMA might inhibit preferentially one of the prevailing voltage-sensitive  $\text{Ca}^{2+}$  channel types in chromaffin cells was further investigated following the strategy depicted in Fig. 5C and D and Fig. 6. Here, the effects of combined applications of PMA and  $\omega$ -agatoxin IVA both on the depolarization-evoked  $[\text{Ca}^{2+}]_i$  transients and  $\text{Mn}^{2+}$  quenching of fura-2 fluorescence were compared with the effects of separate applications of each of the drugs.

Fig. 5C and D shows that combined exposure to  $\omega$ -agatoxin IVA and PMA reduced the peak  $[\text{Ca}^{2+}]_i$  transients to a much larger extent than PMA alone. Furthermore, early  $\text{Mn}^{2+}$  influx recorded in the simultaneous presence of  $\omega$ -agatoxin IVA and PMA was significantly less than in the presence of either  $\omega$ -agatoxin IVA or PMA (Fig. 6). Essentially identical results were obtained by replacing the toxin with 0.3 mM neomycin, which at this concentration appears to block selectively dihydropyridine-resistant channels in chromaffin cells (Duarte et al., 1993b) (data not shown). Taken together, the  $\omega$ -agatoxin IVA data reinforce the hypothesis that PMA inhibits preferentially non-P/Q-type voltage-sensitive  $\text{Ca}^{2+}$  channels. It should be noted, however, that  $\omega$ -agatoxin IVA plus PMA did not produce a significantly larger inhibition of the late phase of the  $[\text{Ca}^{2+}]_i$  transients than PMA alone (Fig. 5D). Nonetheless, a slight inhibitory effect of  $\omega$ -agatoxin IVA could always be observed in individual experiments. The magnitude of the signal measured at this late stage was invariably so small (due to extensive  $\text{Ca}^{2+}$  channel inhibition/inactivation and  $\text{Ca}^{2+}$

buffering) that any true effect of the toxin is likely to be masked by experimental variability. Interestingly and contrary to nitrendipine,  $\omega$ -agatoxin IVA reduced the peak and late (2 min) phases of the  $[\text{Ca}^{2+}]_i$  transients to about the same extent (Fig. 5D, columns labelled  $\omega$ -Aga IVA).

#### 4. Discussion

We have shown that the phorbol ester PMA inhibited depolarization-evoked  $[\text{Ca}^{2+}]_i$  rises in a dose-dependent fashion ( $\text{IC}_{50} = 18 \text{ nM}$ ) in bovine adrenal chromaffin cells. Maximal inhibition, attained at 1  $\mu\text{M}$ , amounted to approximately 45% and 76% at early and late stages of depolarization, respectively. This is larger than the maximal extent of PMA inhibition of ionomycin-evoked  $[\text{Ca}^{2+}]_i$  rises (approximately 23%), also reported in this study. Thus, inhibition of voltage-sensitive  $\text{Ca}^{2+}$  channels is likely to play a major role in the observed reduction of depolarization-evoked  $[\text{Ca}^{2+}]_i$  transients. This conclusion is reinforced by the observation that the phorbol ester concomitantly reduced early depolarization-evoked  $\text{Mn}^{2+}$  influx ( $\text{IC}_{50} = 7 \text{ nM}$ ), a widely accepted mimic of  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$  channels (Merritt et al., 1989).

The ionomycin data indicate that the phorbol ester stimulates intracellular  $\text{Ca}^{2+}$  sequestering and/or extrusion mechanisms in chromaffin cells. In fact, both the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and  $\text{Ca}^{2+}$  pumps have been reported to be stimulated by protein kinase C-mediated processes in different cell types (Balasubramanyam and

Gardner, 1995; Iwamoto et al., 1996). Our observation that PMA failed to affect the time course of the  $[Ca^{2+}]_i$  decay observed in ionomycin (Fig. 3C) suggests that the slight inhibitory effect of ionomycin-evoked  $[Ca^{2+}]_i$  transients might originate primarily from PMA stimulating high-affinity, low-capacity mechanisms such as  $Ca^{2+}$  pumps. PMA does not stimulate the  $Na^+/Ca^{2+}$  exchanger in chromaffin cells (Lin et al., 1994).

Several lines of evidence suggest that the PMA effects reported in our study might reflect protein kinase C stimulation. First, we have demonstrated that staurosporine antagonized the inhibitory action of PMA on depolarization-evoked  $[Ca^{2+}]_i$  transients. Furthermore, preliminary observations from our laboratory indicate that the staurosporine effect is mimicked by other protein kinase C inhibitors, e.g., H-7, bisindolylmaleimide I (Gö 6850) and R0 31-8220. Incidentally, staurosporine (this work) and the latter protein kinase C inhibitors (C.M. Sena and L.M. Rosário, unpublished observations) reduced appreciably the magnitude of the  $[Ca^{2+}]_i$  transients, suggesting that full activation of voltage-sensitive  $Ca^{2+}$  channels requires a critical phosphorylation level as reported by other authors (Carvalho et al., 1998). Secondly, we have previously shown that 4 $\alpha$ -phorbol didecanoate (4 $\alpha$ PDD), a PMA analogue inactive on protein kinase C, did not affect the depolarization-evoked  $[Ca^{2+}]_i$  transients (Sena et al., 1995). Finally, PMA caused maximal inhibitory effects 5 to 10 min after its addition to the cells (this paper and Sena et al., 1995). We have previously reported that, following stimulation with PMA, the *classical* ( $Ca^{2+}$ -sensitive) and *novel* ( $Ca^{2+}$ -insensitive) protein kinase C isoforms present in chromaffin cells (protein kinase C- $\alpha$  and - $\epsilon$ , respectively) translocate from cytosol to membranes along a time course of minutes (the estimated extent of translocation during a 10 min stimulation period was approximately 40% and 75% for protein kinase C- $\alpha$  and - $\epsilon$ , respectively) (Sena et al., 1996).

Bovine chromaffin cells are endowed with several types of voltage-sensitive  $Ca^{2+}$  channels, namely the dihydropyridine-sensitive (L-type) (Cena et al., 1983; Rosario et al., 1989), the  $\omega$ -conotoxin GVIA-sensitive (N-type) (Bossu et al., 1991; Artalejo et al., 1992), the FTX- and  $\omega$ -agatoxin IVA-sensitive (P-type) and the  $\omega$ -conotoxin MVIIC-sensitive (Q-type) (Albillos et al., 1993; Duarte et al., 1993a; Lopez et al., 1994) channels. The latter can also be blocked by  $\omega$ -agatoxin IVA at concentrations above 10 nM (Wheeler et al., 1994). It is uncertain to what extent N-type  $Ca^{2+}$  channels might contribute to high  $K^+$ -mediated  $Ca^{2+}$  influx (Owen et al., 1989; Rosario et al., 1989). Indeed, while several groups including ours have consistently emphasized the lack of effect of  $\omega$ -conotoxin GVIA in depolarization-evoked  $Ca^{2+}$  influx (this paper and Ballesta et al., 1989; Owen et al., 1989; Rosario et al., 1989; Duarte et al., 1993a), others—using single cell  $[Ca^{2+}]_i$  measurements—have reported the occurrence of cell-specific and variable inhibitory effects of the toxin

(Artalejo et al., 1992; Lomax et al., 1997). We take the lack of effect of  $\omega$ -conotoxin GVIA as an indicator that, under the conditions of our study, N-type channels are either inactivated or are potentially capable of undergoing activation in just a rather small proportion of the cells (hence undetectable by toxin exposure). It becomes nonetheless evident that we cannot use  $\omega$ -conotoxin GVIA as a tool for the pharmacological assessment of the role played by N-type  $Ca^{2+}$  channels in the protein kinase C-mediated inhibition of depolarization-evoked  $Ca^{2+}$  influx. Adding to this difficulty is the fact that a sizeable component of depolarization-evoked  $Ca^{2+}$  influx appears to be resistant to cocktails of specific channels blockers, as evidenced by us (this paper) and others (Albillos et al., 1993; Lopez et al., 1994; Currie and Fox, 1997) using different techniques. Whether this might originate from the operation of an as yet uncharacterized fourth type of voltage-sensitive  $Ca^{2+}$  channels or from insufficient blockade of the known channels by the different toxin and non-toxin blockers remains to be clarified.

Our experiments suggest that PMA reduces the depolarization-evoked  $[Ca^{2+}]_i$  transients and  $Mn^{2+}$  quenching of fura-2 fluorescence by inhibiting preferentially L-type  $Ca^{2+}$  channels over P/Q type channels. This is because: (1) nitrendipine did not affect significantly the PMA-resistant component of depolarization-evoked  $Ca^{2+}$  influx. The fact that, in some experiments, nitrendipine plus PMA produced a larger inhibition than PMA alone may be interpreted assuming that the phorbol ester did not always suppress  $Ca^{2+}$  influx through L-type channels, in keeping with the situation found in other cell types (Haymes et al., 1992; Pemberton and Jones, 1997); (2) in contrast,  $\omega$ -agatoxin IVA inhibited strongly the PMA-resistant component of  $Ca^{2+}$  influx recorded at the onset of depolarization, regardless of whether the influx was assessed by  $[Ca^{2+}]_i$  measurement or  $Mn^{2+}$  quenching of fura-2 fluorescence. This is consistent with PMA leaving largely intact  $Ca^{2+}$  influx through P/Q-type  $Ca^{2+}$  channels; and (3) similar to nitrendipine, PMA inhibited the late phase of the  $[Ca^{2+}]_i$  transients more severely than the peak. The fact that the inhibitory action of nitrendipine increases with time suggests that, compared to the dihydropyridine-insensitive  $Ca^{2+}$  channel subtypes, L-type channels might be less prone to undergo long-term  $Ca^{2+}$ -dependent rundown and/or inactivation processes, previously demonstrated to occur in other cell types (for review see Lnenicka and Hong, 1997). If this were indeed the case, the fact that  $\omega$ -agatoxin IVA reduced the peak and late phases of the  $[Ca^{2+}]_i$  transients to about the same extent might be taken to indicate that putative dihydropyridine-,  $\omega$ -conotoxin GVIA- and  $\omega$ -agatoxin IVA-resistant  $Ca^{2+}$  channels have the highest susceptibility to the above  $Ca^{2+}$ -dependent rundown/inactivation processes. This, however, can only be tested by resorting to patch-clamp techniques for a more effective isolation of specific  $Ca^{2+}$  channel subtypes. Our conclusion that dihydropyridine-sensitive  $Ca^{2+}$  chan-

nels are under strong negative control by protein kinase C-mediated phosphorylation is consistent with the observation (Kley, 1988; Sena et al., 1995) that PMA inhibits strongly the  $[Ca^{2+}]_i$  transients induced by Bay K 8644, a specific L-type  $Ca^{2+}$  channel agonist (Garcia et al., 1984). L-type channels appear to be a consistent target for the inhibitory action of protein kinase C activators in several cell types (Haymes et al., 1992; Bouron et al., 1995; Pemberton and Jones, 1997; Zhang et al., 1997).

Consistent with the results described in this study, we have previously reported that PMA inhibits high  $K^+$ -evoked catecholamine release from bovine chromaffin cells (23–27% inhibitions at 0.1–1  $\mu M$ ) (Sena et al., 1995). Phorbol esters have also been shown to inhibit catecholamine release evoked by a range of other secretagogues that may use activation of voltage-sensitive  $Ca^{2+}$  channels as the primary route for chromaffin cell activation (e.g., nicotine,  $Ba^{2+}$ , veratridine and scorpion venom) (Wilson, 1990). In contrast, phorbol esters enhance ionomycin (Pocotte et al., 1985)- and calcium (Lee and Holz, 1986)-evoked catecholamine release from intact and permeabilized bovine chromaffin cells, respectively, apparently by increasing the  $Ca^{2+}$  sensitivity of key proteins involved in exocytosis (Lee and Holz, 1986). The simplest model to account for these differences is that protein kinase C activators have antagonistic consequences on the early and late steps of secretion, with the inhibition overcoming the activation of the exocytotic machinery that occurs downstream the activation of  $Ca^{2+}$  channels. Based on this model, phorbol esters might be expected to either potentiate the secretion evoked by other non-depolarizing secretagogues or to have variable effects (including no detectable effect) on the secretion evoked by mixed agonists (e.g., acetylcholine and carbachol). This was indeed observed (Burgoyne and Norman, 1984; Morita et al., 1985).

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